

Attached hereto is a marked-up version of the changes made to the specification and claims by the current amendment. The attached page is captioned "Version with markings to show changes made".

AMENDMENTS

In the Specification:

Please amend the paragraph on page 1 commencing at line 5, as follows:

--This patent application claims priority to U.S. provisional patent application Serial No. 60/161,703, filed 27 Oct. 1999, which is incorporated herein by reference.--

Please amend the paragraph on page 56, commencing at line 9, as follows:

--Figure No. 3 shows the comparison of *in vivo* acyl-CoA levels in BL21(DE3) *panD* strains with and without mm-CoA mutase. For each CoA, the ratio of the amount in the strain containing the mutase to the amount in the control strain was determined. Interestingly, malonyl-CoA was increased about 25-fold and succinyl-CoA about 3-fold. Acetyl-CoA and CoA were increased just slightly, and propionyl-CoA was not detected in either case.--

Support for this paragraph is found in the application on page 56, lines 9-14.

Please amend the paragraph on page 61, commencing at line 7, as follows:

--Figure No. 4 shows the comparison of *in vivo* acyl-CoA levels with and without the mutase and with and without hydroxocobalamin. In the cells over-expressing the mutase and grown with hydroxocobalamin, methylmalonyl-CoA comprised 13% of the overall CoA pool, whereas in the other cells no methylmalonyl-CoA was detectable. The background level of counts is about 0.25% of the overall number of counts in the CoAs, suggesting that any methylmalonyl-CoA present in *E. coli* strains not over-expressing the mutase would comprise at most 0.25% of the overall CoA pool, or 2% of the amount of methylmalonyl-CoA observed in the strain over-expressing the mutase. The composition of the CoA pool observed for the *E. coli*

pamD strain is consistent with that observed previously for *E. coli pamD* mutants grown on glucose.--

Support for this paragraph is found in the application on page 61, lines 7-17.

Please amend the paragraph on page 62, commencing at line 2, as follows:

Figure 4 shows the results of CoA analysis of *E. coli* over-expressing methylmalonyl-CoA mutase. The levels of ^3H detected in fractions collected from HPLC of cell-free extracts from ^3H β -alanine-fed *E. coli* harboring either the pET control vector grown without hydroxocobalamin (solid line), pET grown with hydroxocobalamin (short dash), pET over-expressing the mutase and grown without hydroxocobalamin (long dash), or pET over-expressing the mutase and grown with hydroxocobalamin (medium dash) are shown.--

Support for this paragraph is found in the application on page 62, lines 2-8.

Please amend the paragraph on page 81, line 17 through page 82, line 3, as follows:

As described in Example 1, the translationally coupled genes, *mutA* and *mutB*, encoding the β - and α -subunits of methylmalonyl-CoA mutase from *Propionibacterium shermanii*, were amplified by PCR and inserted into an *E. coli* expression vector containing a T-7 promoter. The naturally occurring GTG start codon for *mutB* was changed to ATG to facilitate expression [5]. Heterologous expression of the mutase genes in media containing [^3H] β -alanine and the adenosylcobalamin (coenzyme B₁₂) precursor, hydroxocobalamin, yielded active methylmalonyl-CoA mutase. HPLC analysis of extracts from *E. coli* BL21(DE3)/*pamD* harboring the mutase genes indicated production of methylmalonyl-CoA, which comprised 13% of the intracellular CoA pool (shown in Figure 6). This work demonstrates that one can introduce a biosynthetic pathway for an important PKS substrate into a heterologous host, and that one can measure the intracellular concentration of acyl-CoAs. In accordance with the present invention, the methylmalonyl-CoA mutase gene (*shm*) from *E. coli*, which has codon usage closer to yeast and encodes a single polypeptide [16], can also be employed.--

Support for this paragraph is found in the application on page 81, lines 17 to page 82, line 1.

Please amend the paragraph on page 83, commencing at line 1, as follows:

--Propionyl-CoA is not detected in *E. coli* SJ16 cells grown in the presence of [³H] β-alanine with or without the addition of propionate in the growth media. When *E. coli* SJ16 cells were transformed with a pACYC-derived plasmid containing the *Salmonella typhimurium* propionyl-CoA ligase gene (*prpE*) under the control of the *lac* promoter, a small amount of propionyl-CoA was observed (~0.2% of total CoA pool) in cell extracts. When 5 mM sodium propionate was included in the culture medium, about 14-fold more propionyl-CoA was produced (~ 3% of the total CoA pool).--

Support for this paragraph is found in the application on page 83, lines 1 to 9.

Please amend the paragraph on page 85, commencing at line 5, as follows:

--Genes involved in the production of substrates (e.g. methylmalonyl-CoA and/or propionyl-CoA), and the *yfp* gene can preferably be stably integrated into the yeast chromosome in appropriate copy number to produce adequate levels of desired acyl-CoAs and post translational PKS modifications. Genes can first be introduced into the intermediate Bluescript cloning vector as described. Then, the fragment containing the promoter-gene-terminator cassette can be transferred as a L1-L3 fragment to a yeast "delta integration" vector [18] [19] that allows chromosomal integration of the cassettes into one or more of the ca. 425 delta sequences dispersed throughout the yeast chromosome (see Figure 9). These vectors have cloning sites compatible with those in the L1-L3 linkers to permit direct transfer of promoter-gene-terminator cassettes as L1-L3 fragments. They also contain the excisable Ura3 selection marker flanked by two bacterial *hisG* repeats ("URA Blaster"), enabling insertion of multiple identical or different genes into the yeast chromosome by repetitive integration's. After selection for gene integration on media lacking uracil, the Ura3 gene fragment is removed by selecting for marker loss via

excisional recombination by positive selection with 5-fluoroorotic acid (FOA), which renders the Ura3 gene toxic to yeast. This enables the introduction of stable pathways needed for acyl-CoA precursors and Sfp into yeast, while conserving the Ura marker to allow its subsequent use in plasmids containing other genes.--

Support for this paragraph is found in the application on page 85, lines 5-24.

Please amend the paragraph on page 85, line 25, through page 86, line 7, as follows:

--The single-gene mutase, Sbm (Sleeping beauty mutase), from *E. coli* [16], can be cloned as follows. Primers designed based on the DNA sequence were used to PCR amplify the *sbm* gene from *E. coli* genomic DNA as a *Nde*I-L2 fragment. The general strategy for cloning the genes into yeast expression vectors follows that of Kealey *et al.* [3] (see Figure 9). One can first clone the genes as *Nde*I-L2 fragments into the intermediate Bluescript cloning vector. The promoter-gene-terminator cassette can then be excised as an L1-L3 fragment, transferred to the yeast integrating vector, restricted with L1/L3, and introduced into the yeast chromosome as described above. As an alternative to Sbm, one can use the two-gene mutase from *P. shermanii*; the translationally coupled genes have each been amplified by PCR as *Nde*I-L2 fragments and can be integrated into yeast as described above.--

Support for this paragraph is found in the application on page 85, line 25 to page 86, line 7.

Please amend the paragraph on page 86, commencing at line 14, as follows:

--The *pccB* and *accA1* genes involved in the propionyl-CoA carboxylation pathway in *S. coelicolor* can be amplified by PCR from genomic DNA. As shown in Figure 9, the genes can be cloned into the intermediate Bluescript vector between *Nde* I and L2, then transferred to the yeast integrating vector via L1/L3. One can express the *S. coelicolor* genes shown to be effective in *E.*